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ARTICLE

^{1}H, ^{13}C and ^{15}N NMR assignments of Duck HBV primer loop of the encapsidation signal epsilon

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Abstract The replication of the hepatitis B virus is initiated by binding of the viral reverse transcriptase protein complex to the apical stem loop of the epsilon element to place it next to the primer loop, from which a four nucleotide DNA primer is subsequently synthesized. Here, we present the $^{1}H/^{13}C/^{15}N$ NMR assignments of the bases and sugars of the 37 residues primer loop of Duck HBV epsilon (BMRB-entry 15786).

Keywords Hepatitis B virus (HBV) · RNA · Duck · Epsilon · Primer loop

Biological context

The hepatitis B virus is a member of the hepadnavirae (Beck and Nassal 2007). It has a small genome of 3.2 kb partially double-stranded DNA, which is replicated after infection via a pregenomic RNA (pg-mRNA) intermediate. The pg-mRNA is encapsidated into immature core particles together with the HBV reverse transcriptase (RT). Binding of the RT protein complex to the apical stem-loop of the encapsidation signal, epsilon (ε), a conserved 60 nt RNA element located at 5′-end of the pg-mRNA, initiates encapsidation (Beck and Nassal 2007; Girard et al. 2007). After binding of this complex, a 4 nt DNA primer is synthesized from the internal primer loop of the epsilon signal (Beck and Nassal 2007; Flodell et al. 2006). The resulting complex translocates to a 3′-proximal primer binding site, from where the pg-mRNA is reverse transcribed into the genomic DNA.

More than 350 million people are chronically infected by human HBV (Lee 1997). No effective treatment exists as yet for chronic infection. Knowledge of the structural details of the HBV replication, specifically of ε and RT-ε complex, is therefore of crucial importance, for development of anti-virals. Although the replication of human HBV is understood in some detail many questions remain, due to the lack of fully functional in vitro replication system. Many of the molecular details of HBV replication have instead been worked out from the similar but not identical Duck HBV, for which a fully functional in vitro replication system does exist (Beck and Nassal 2007). We study both the Duck and Human HBV epsilon in parallel and present here the results on the Duck ε primer loop.

Methods and experiments

The RNA sequence was produced as described earlier by Girard et al. (2007). NMR samples of non-labelled RNA with strand concentration of 0.19 mM were prepared in H_2O (10% D_2O) and in D_2O in phosphate buffer (10 mM sodium phosphate pH 6.7, 0.1 mM EDTA); in addition a uniformly $^{13}C/^{15}N$ labelled sample with 1.0 mM RNA strand concentration was prepared in H_2O (10% D_2O) in the same buffer (Girard et al. 2007).

Sodium-2, 2-dimethyl-2-silapentane-5-sulphonate (DSS) was added (1.5 mM) to the labelled sample for calibration of ^{1}H resonances; for unlabelled samples this served as an external reference; ^{13}C and ^{15}N were indirectly calibrated from the ^{1}H resonances. All experiments were recorded on a Varian Unity INOVA.

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Standard methods (Flinders and Dieckman 2006; Wijmenga and van Buuren 1998) were used to obtain ^{1}H resonance assignments and subsequently extended using heteronuclei via (^{1}H, ^{13}C/^{15}N) correlation experiments. For this, 500 MHz (^{1}H, ^{1}H) NOESY spectra with a 250 ms mixing time were recorded on the non-labelled H_2O sample at 5 and 15°C. In addition, 800 MHz (^{1}H, ^{1}H) NOESY spectra with mixing times of 30 and 500 ms were recorded on the non-labelled D_2O sample at 15 and 25°C. Furthermore, an 800 MHz DQF-COSY was recorded at 25°C (non-labelled D_2O sample) to obtain H5–H6 correlations and sugar puckering from H1′ to H2′ correlations (Girard et al. 2007). To obtain ^{13}C resonance assignments, a set of 600 MHz (^{13}C, ^{1}H) HSQC spectra, for the regions C6/H6/C8/H8 (Fig. 1), C2H2, C5/H5 and C1′/H2′, were recorded in H_2O at 15 and 25°C (uniformly labelled sample). To extend the H1′/H2′/C1′ sugar resonance assignments to H3′/H4′/C2′/C3′/C4′ a 600 MHz a modified 3D HCCH-TOCSY was recorded at 25°C in H_2O (uniformly labelled sample) (Ampt, Tessari, and Wijmenga, personal communication) and combined with a 600 MHz 2D H1′(C1′)C2′-COSY, which correlates H1′ and C2′ within the same residue, to distinguish C2′ from C3′ signals. To assign C4 of uridine and C6 of guanine residues from the imino protons a 600 MHz 2D H1/3(N1/3)C4/6 (U/G) experiment was recorded at 15°C in H_2O (uniformly labelled sample) and subsequently connected to the H5 of uridine via a 600 MHz 2D H5(C5)C4 experiment. The latter experiment also provides H5/C4 correlations in cytosines. Finally, to assign the C2 of uridine and guanine residues a 600 MHz 2D H1/3(N1/3)C2 (U/G) experiment at 15°C in H_2O (uniformly labelled sample) was employed. The H1/3(N1/3)C2 (U/G) and H1/3(N1/3)C4/6 (U/G) are modified versions of the well known HNCO experiment. To assign N1/3 (U/G) nuclei from imino protons a 600 MHz ^{15}N, ^{1}H-HSQC spectra were recorded at 5 and 15°C in H_2O

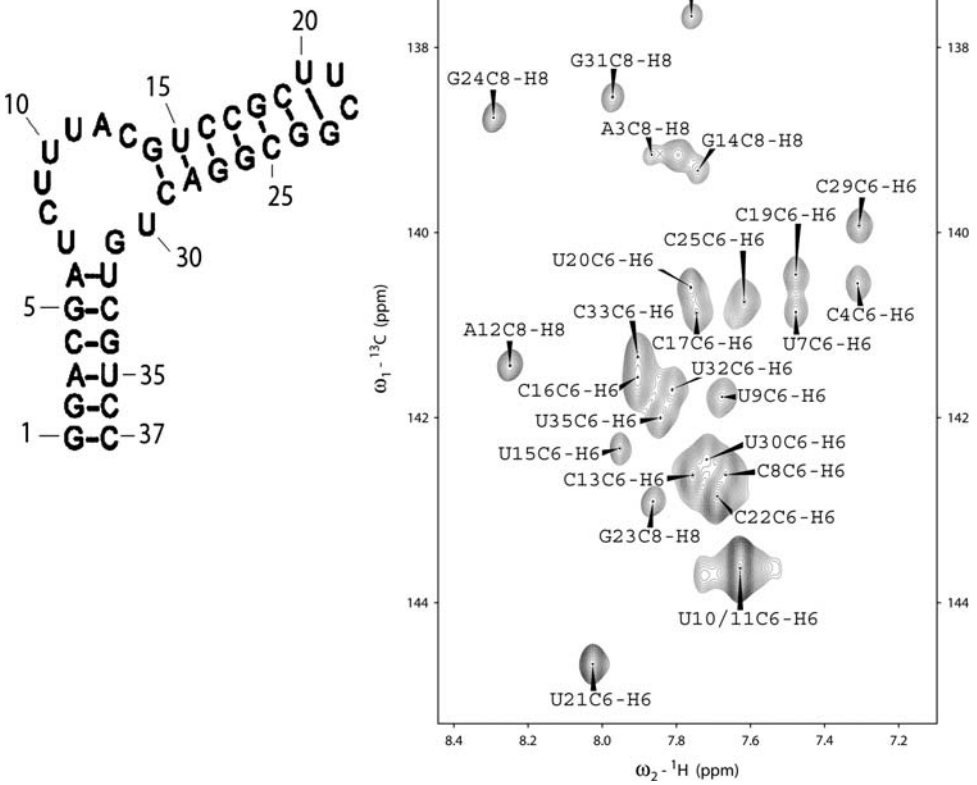

Fig. 1 2D ^{1}H/^{13}C CT HSQC spectrum recorded at 15°C at 600 MHz on the 1.0 mM sample of the fully ^{13}C/^{15}N labelled (10 mM sodium phosphate pH 6.7, 0.1 mM EDTA, 1.5 mM DSS, 90:10 H_2O/D_2O) primer loop of Duck HBV. Resonance assignments are indicated by residue type and sequence number followed by atom names. Shown are two regions of the C6H6/C8H8 spectra. On the left the secondary structure of the primer loop is shown. Overlap between U10 and U11 is shown in both the C6 and H6 resonance position

(uniformly labelled sample). N1 (U/C) and N9 (G/A) nuclei could be assigned using a 600 MHz 3D H1′C1′N1/9 experiment recorded at 25°C on the same sample. The heteronuclear experiments mentioned above are (slightly) modified versions of original experiments (see e.g., reviews by Wijmenga and van Buuren 1998; Flinders and Dieckman 2006 and references therein).

Spectra were processed using NMRPipe (Delaglio et al. 1995) and resonance assignment was performed with Sparky software (Goddard and Kneller 2001).

Assignments and data deposition

The assignments at three different temperatures, 5, 15 and 25°C have been deposited into the BMRB databank (accession number 15786). The 250 ms (^{1}H, ^{1}H) NOESY in H_2O at 15°C was recorded to establish the number of base pairs in the RNA molecule and assign the imino proton resonances following sequential walks. All expected imino resonances were observed except for the imino resonances of the potential U7:G31 and A6:U32 at the top of lower stem (Fig. 1, left); as expected the imino resonances in the internal loop (U7-C13; U30) and for U21 in the UUCG hairpin loop were also not observed (Fig. 1, left). The H2 resonances were also assigned from the 250 ms (^{1}H, ^{1}H) NOESY and later completed/confirmed employing (^{13}C, ^{1}H) HSQCs. Resonances of the other non-exchangeable protons were subsequently assigned via sequential walks (H1′–H6/8/2; H6/8/2–H6/8/2; H5–H6; H2′–H6/8/2) in the 30 and 500 ms (^{1}H, ^{1}H) NOESY spectra in D_2O combined with (^{1}H, ^{1}H) DQF-COSY for the intra-residue H5–H6 correlations. The 500 ms NOESY was used to assign the H1′ protons and H2/H5/H6/H8 protons and the 30 ms NOESY for H2 and H5/H6/H8 protons. Thanks to the long mixing time (500 ms) H1′–H6/8, H6/8–H6/8 H2′–H6/8) the sequential walks were never completely interrupted. The near complete assignments (see below) of the imino, base and H1′ protons were extended via J-correlated experiments (see "Methods and experiments") to the heteronuclei C8/C6/C5 and C2 resonances, N1/3 (G/U), C2 (U), C6 (G), N1/9 (U/G). Further assignments of sugar protons and carbons were achieved using the 3D HCCH-TOCSY and 2D H1′(C1′)C2′-COSY; the assignment of the H$x'$and Cx' ($x = 1'$–$4'$) were nearly complete in the non-helical regions, while resonances of helix residues were less complete due to the strong overlap (see below); assignment of C5′ and H5′/H5″was not attempted as these resonances form a CH_2 moiety and were deselected by the delay settings in the 3D HCCH-TOCSY.

In summary, in the helices all imino proton resonances were assigned as well as their J-correlated N1(G) and N3(U) resonances, only imino proton resonances of base-pair A6:U32 and U7:G31 were not observed. A complete assignment of the aromatic protons was obtained, except for the H5 resonance of C36. All C6/8 (A/G) are assigned except for the stem residues G1 C8, A6 C8, G26 C8, C36 C6 and C37 C6 and the internal loop residue A12. All C2(A) are assigned and 9 out of 10 C4 (U) resonances, 9 out of 11 C6 (G) and half the C2(U/G) resonances. The H6 and C6 resonances of U10 and U11 essentially overlap (Fig. 1, here these resonances overlap, whereas in NOESY spectra the resonances show a slightly different position). The N1 (U) and N9 (G) resonances are all assigned, except for N1 for U11, and N9 for G14, G27 and G34. The unambiguous assignments of the sugar resonances (see above), Hx' and Cx' ($x = 1'$–$4'$), are nearly complete in the non-helical regions defined as A6-C13,U20-G24 and U30-U32 (H1′ 100%; C1′ 69%; H2′ 100%, C2′ 63%, H3′ 69%, C3′ 63%, H4′ 63%, C4′ 63%), while the unambiguous resonance assignment of helix residues is less complete due to the strong overlap, except for H1′ and H2 ($\sim$100%). Assignment of C5′ and H5′/H5″was not attempted as described above.

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